Modulation of the TRAIL apoptotic pathway to optimize chemoradiation in preclinical models of cervical cancer

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SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

SUMMARY

Today cervical cancer is still the most common cancer prevailing among women in developing countries, while in developed countries incidence has been declining since the implementation of screening programs. Infection with high risk human papillomavirus (HR HPV) is the main risk factor involved in cervical carcinogenesis, with HPV16 and 18 being responsible for about 70% of cervical neoplasia. The HPV DNA test is positive in most cervical cancer cases and high grade cervical intraepithelial neoplasias.

Locally advanced disease is treated with chemoradiation. This treatment results in 66-79% 5-year survival. Further escalation of chemoradiation doses to increase treatment efficacy is limited by the small therapeutic window. Novel targeted agents with only partly overlapping toxicity profile are therefore considered to be of interest to be tested.

Chemoradiation-induced DNA damage leads to p53-mediated apoptosis. However, in cervical cancers most tumors are HPV-positive and the HPV E6 viral oncogene expression disrupts normal functioning of p53, thereby decreasing chemoradiation efficacy. Unlike other HPV-negative epithelial cancers, HPV-positive cervical cancers express wild-type p53. Therefore agents that can tackle the consequences of HPV infection and restore p53 functionality are of potential interest for cervical cancers. Additionally, the extrinsic apoptotic pathway offers an alternative route to improve treatment since this pathway does not need p53 in its execution. Cytokines belonging to the tumor necrosis factor (TNF) family like TNF, TNF-related apoptosis inducing ligand (TRAIL) and Fas ligand, can induce apoptosis by binding to their cell membrane receptors. Of these pathways the TRAIL-pathway is of interest as target as it induces apoptosis selectively in tumor cells but not in normal cells.

This thesis focuses on exploring the potential of death receptor 4 (DR4) or 5 (DR5) targeting ligands and drugs as anticancer agents to enhance cervical cancer treatment efficacy.

Following a short introduction in **chapter 1**, an overview of potential new drugs to be combined with chemoradiation in HPV positive cervical cancer is presented in **chapter 2**. We focused on targeted anticancer drugs aimed at eliminating the consequences of HR HPV E6 and E7 activity. Strategies for direct and indirect targeting of HR HPV E6 and E7, including RNA interference, small molecules, proteasome inhibitors, and histone deacetylase inhibitors, are described. In addition, the extrinsic apoptosis pathway as possible alternative therapeutic target for apoptosis induction is reviewed. The rational for implementing recombinant human TRAIL and DR4/DR5-agonistic antibodies and the latest developments in combining these drugs with standard treatment in preclinical settings as well as clinical trials are discussed.

In **chapter 3** the role of high risk HPV16 E6 was investigated by selective silencing of E6 RNA in order to restore p53 functionality and sensitize the resistant HPV16-positive cervical cancer cell line SiHa to apoptosis by cisplatin, irradiation, agonistic anti-Fas antibody or recombinant human (rh)TRAIL. E6 siRNA decreased E6 mRNA levels by approximately

50%. E6 siRNA resulted in enhanced p53 expression and p21 upregulation, demonstrating restoration of p53 functionality in SiHa, without inducing high levels of apoptosis (less than 10%). Cell surface expression of the proapoptotic TNF receptor family members Fas, DR4 and DR5 was not affected by E6 suppression. In combination with cisplatin, E6 suppression conferred susceptibility to cisplatin-induced apoptosis. E6 suppression, however, had no effect on apoptosis-induction by irradiation, anti-Fas antibody or rhTRAIL alone. Especially, cisplatin in combination with rhTRAIL or anti-Fas antibody induced high apoptosis levels in E6 suppressed cells. At the molecular level, cisplatin treatment resulted in elevated p53 levels, enhanced caspase-3 activation and reduced p21 levels in E6 suppressed cells. Cisplatin in combination with DR-ligands enhanced caspase-8 as well as caspase-3 activation and reduced XIAP levels in these cells. The effects were not due to cisplatin-induced upregulation of DR membrane expression in E6 suppressed cells.The enhanced apoptosis in E6-supressed cells was related to reduced XIAP levels and not due to reduced p21 expression. In conclusion, targeting E6 or XIAP in combination with cisplatin can efficiently potentiate rhTRAIL or anti-Fas antibody induced apoptosis in HPV-positive cervical cancer cells.

A plausible approach to diminish the effect of high risk HPV E6 on p53 degradation is utilization of the clinically available proteasome inhibitor bortezomib. Earlier our group showed that the proteasome inhibitor MG132 sensitized cervical cancer cells to rhTRAIL. However, the role of rhTRAIL-induced selective receptor activation in cervical cancer cells is unclear and the mechanism by which proteasome inhibition enhances rhTRAIL-induced cell death in cervical cancer cells needs to be further elucidated. Therefore, in **chapter 4** we showed for the first time that bortezomib enhances cell death in cervical cancer cells following agonistic DR4/DR5 antibody treatment through distinct apoptotic pathways. Following DR4 activation bortezomib enhances apoptosis in SiHa cells by increasing caspase-8 and -3 activation and c-FLIP and XIAP cleavage. Bortezomib-enhanced apoptosis via DR5, however, requires mitochondria-mediated events through p53-dependent Bax induction, independent of Bid. In intermediate TRAIL sensitive HeLa cells, which inherently require mitochondrial amplification to induce apoptosis, bortezomib treatment following DR4 activation enhanced apoptosis by increasing caspase-8 and -3 activation and c-FLIP and XIAP cleavage. Bortezomib-enhanced apoptosis in HeLa, following DR5 activation, proceeds via the mitochondrial apoptotic pathway through Bid cleavage, independently of p53 and Bax. In both cell lines the sensitizing effects of bortezomib on DR4 activation, but not DR5 activation, can be mimicked with c-FLIP and XIAP RNA interference. The clinical implication of activating distinct apoptotic pathways via DR4/DR5 should be accounted for when selecting chemotherapeutic or targeted agents for combination therapies to enhance the efficacy of DR4/ DR5 selective targeted drugs.

In addition to rhTRAIL, newer TRAIL ligand variants that specifically target DR4 or DR5 are available. Targeting a single receptor may enhance apoptosis effect since the effects of DcRs are evaded and the effect may increase when the ligand has a higher affinity for the specific DR.

In **chapter 5** the potential of enhancing irradiation efficacy with rhTRAIL variants *in vitro* and in mice bearing a bioluminescent human cervical xenograft model are described.Irradiation strongly augmented DR5 membrane expression in HeLa and SiHa but not in CaSki cells, therefore DR5-selective rhTRAIL (D269H/E195R) was used next to wild type rhTRAIL to compare their efficacy either alone or combined with irradiation. In combination with irradiation both D269H/E195R and rhTRAIL induced a 2-fold stronger increase in apoptosis and cell death in HeLa cells, while a smaller effect was observed in CaSki and SiHa cells. Single cell FRET analyses demonstrated a much faster onset and a strongly enhanced rate of caspase-8 activation by D269H/E195R or rhTRAIL in HeLa-ICRP cells pre-treated with irradiation compared to single treatment with ligand. In addition, D269H/E195R more efficiently activated caspase-8 compared with rhTRAIL, also when combined with irradiation. This was reflected in a faster induction of caspase-3 activation and PARP cleavage. In HeLa-luc xenografts, combination therapies inhibited tumor growth stronger than irradiation or ligand alone, and was most pronounced for irradiation plus D269H/E195R. At the end of treatment (day 10), irradiated tumors were 43% smaller when rhTRAIL was added ($P = 0.021$) and 81% smaller in combination with D269H/E195R ($P = 0.034$) when compared with irradiation alone. Irradiation sensitized cervical cancer cells and xenografts preferentially to the DR5-selective TRAIL variant D269H/E195R.

Another alternative to specifically target the DR4 or DR5 are the agonistic human DR4/DR5-monoclonal antibodies. In patients agonistic DR4 and DR5-antibodies have a longer half-life time (about 18 days) compared to rhTRAIL (21-43 minutes). In **chapter 6** the effect of irradiation combined with the agonistic DR4-agonistic antibody mapatumumab on *in vitro* and *in vivo* cervical cancer models was described. Mapatumumab is effective in a range of solid and hematological cancer models in vitro and in animal studies, including cervical cancer cells. However, no preclinical animal studies data are available for the potential interesting clinical setting of mapatumumab with irradiation in cervical cancer. We therefore explored the effect of irradiation together with mapatumumab in the cervical cancer cell lines HeLa, SiHa and CaSki. Both irradiation and mapatumumab decreased cell viability in HeLa cells, which was further enhanced by combining these treatments. Irradiation pre-treatment did not enhance mapatumumab cytotoxicity in CaSki and SiHa cells. Using FRET, we observed a 2-fold faster onset and an enhanced rate of caspase-8 activation in HeLa cells treated with irradiation plus mapatumumab compared to mapatumumab alone, while irradiation did not induce caspase-8 activation. The combination of irradiation plus mapatumumab showed the strongest anti-tumor activity in HeLa-luc xenografts. On day 16, when the first mice had to be sacrificed, tumors were up to 90% smaller in the irradiation plus mapatumumab treated group compared to the irradiation group ($P = 0.021$) and the mapatumumab group ($P = 0.007$). A similar pattern was observed using the bioluminescent signal as a read-out for response. Moreover, the combination of irradiation and mapatumumab strongly enhanced median survival of mice to > 36 days compared with 24 days for mapatumumab.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

In the history of establishing HPV as the main etiologic agent of cervical cancer, there is a pre- and post-zur Hausen era. Before zur Hausen's isolation of HPV16 and 18 from cervical carcinoma specimens in the 1980s, the herpes virus was considered as the culprit of cervical carcinogenesis (1). More than two decades later, zur Hausen's discovery has changed the paradigms of controlling cervical cancer on different fronts, from screening, treatment, to prophylactic vaccine development.

Targeting HR-HPV E6

Given the fact that the majority of cervical cancers retain HPV infection, several studies addressed the potential of targeting HPV in cervical cancer treatment. In **chapter 2** the strategies aimed at potentially targeting HPV-positive cervical cancers were discussed. The tumor suppressor gene p53 is the main target of HR HPV E6, therefore persistent infection reduces cervical cancer response to genotoxic insult. E6 suppression as well as cisplatin is able to increase p53 expression (**chapter 3**). However, the E6 siRNA-mediated p53 upregulation is only temporary. This is probably due to the short-term E6 downregulation by siRNA method. It has been reported that cisplatin prolongs p53 stabilization in E6-suppressed cells. Additionally cisplatin is able to decrease E6 mRNA levels in HPV-positive cervical cancer cells. This can explain our finding in **chapter 3** that the combination of E6 siRNA and cisplatin enhance cell death in cervical cancer cells. However, the application of siRNA is not yet ready for clinical application. From the described HPV targeting approaches that restore p53 functionality in cervical cancer cells, proteasome inhibitor bortezomib and HDAC inhibitors are currently in clinical trials. The HDAC inhibitor valproate together with the DNA methylation inhibitor hydralazine in combination with cisplatin and topotecan has entered a phase III clinical trial in cervical cancer patients. The preliminary results showed extended progression free survival in patients treated with the novel combination therapy (2). Drugs targeting DR4 and DR5 have entered phase I-II clinical trials with patients with solid and hematological malignancies (3-12). So far the best tumor responses were reported in non-Hodgkin lymphoma patients treated with the DR4-agonistic antibody mapatumumab, namely complete response in two patients with follicular lymphoma (3).

Targeting c-FLIP and XIAP

Our results in chapter 3 and 4 put forward XIAP and c-FLIP as interesting therapeutic targets to enhance apoptosis in cervical cancer cells. It remains unknown, however, whether inhibition of XIAP and/or c-FLIP is sufficient to sensitize cervical cancer to chemoradiation. In **chapter 5** and **6**, we have shown that drugs targeting DR4 and DR5 were potentiating the effect of irradiation. In **chapter 4** we describe that c-FLIP and XIAP are crucial determinants in rhTRAIL-induced apoptosis. Especially DR4-mediated apoptosis could be enhanced by

suppressing c-FLIP and XIAP expression. Therefore, it may be even more effective to combine chemoradiation with drugs inducing DR4 apoptosis signaling and drugs targeting intracellular key components of the apoptosis pathway, i.e. c-FLIP and/or XIAP. Instead of c-FLIP and/or XIAP inhibitors, bortezomib might be used, since it highly effectively sensitized all cervical cancer cell lines to both DR4 and DR5 mediated apoptosis.

It has been reported that high XIAP expression was associated with more aggressive behavior and poor tumor differentiation in squamous cervical intraepithelial neoplasia and carcinoma. In addition, the efficacy of chemotherapeutic drugs in inducing apoptosis was correlated with their ability to decrease XIAP expression. These data indicate that a clinical trial with XIAP inhibitors in cervical cancer patients deserves attention. Several early phase clinical trials with XIAP inhibitor AEG35156 are ongoing in solid as well as hematological tumors. The first results showed that AEG35156 was well tolerated and induced dose-dependent decrease of blood XIAP mRNA and XIAP protein levels (13-15).

C-FLIP upregulation is associated with HR-HPV positive status and malignant transformation, being predominantly expressed in CIN2, CIN3 and cervical squamous cell carcinoma (16, 17). So far direct inhibition of c-FLIP is not possible, therefore indirect targeting via the prosurvival pathways that induce c-FLIP expression have been exploited, like nuclear factor kappa-B (NF-κB) pathway, epidermal growth factor receptor (EGFR) signaling pathway and COX-2 pathway. A broad range of drugs has been reported to decrease c-FLIP levels, like proteasome inhibitors, EGFR inhibitors and HDAC inhibitors (18). Especially EGFR may be an interesting target in cervical cancer to indirectly affect c-FLIP levels, since increased expression of EGFR is associated with worse prognosis in irradiated cervical cancer (19). The EGFR tyrosine kinase inhibitor erlotinib was tolerated up to 150 mg/day in combination with chemoradiation in stage IIb-IIIb patients (20). In a phase II clinical trial performed by the same group, the addition of erlotinib showed potential interesting antitumor effect (21). Unfortunately, the effect of erlotinib on c-FLIP levels in these cervical cancer patients has not yet been monitored. Another interesting drug to consider is a fusion protein targeting EGFR and DR, thereby decreasing c-FLIP levels and enhancing apoptosis induction (22, 23). Whether this fusion protein will enter a clinical trial remains to be seen.

CONCLUSIONS

The data in this thesis demonstrate that utilizing of DR4- and DR5-mediated pathway may enhance chemoradiation efficacy in cervical cancer patients. The effects of these agents might potentially be enhanced by a proteasome inhibitor. Our preclinical animal data showed the superiority of adding the DR4 agonistic antibody mapatumumab to irradiation compared to rhTRAIL or a DR5-selective TRAIL. Further exploration of mapatumumab combined with chemoradiation in the clinical setting is therefore recommended.

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